PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 10 May 2001 (10.05.01)	
International application No. PCT/EP00/00455	**Applicant's or agent's file reference** 1498PTWO
International filing date (day/month/year) 21 January 2000 (21.01.00)	**Priority date** (day/month/year) 04 February 1999 (04.02.99)
Applicant PIZZARIELLO, Andrea et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 August 2000 (30.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Zakaria EL KHODARY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

EP0000455

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/00

A1

(11) International Publication Number: WO 00/46393

(43) International Publication Date: 10 August 2000 (10.08.00)

(21) International Application Number: PCT/EP00/00455

(22) International Filing Date: 21 January 2000 (21.01.00)

(30) Priority Data:
MI99A000210 4 February 1999 (04.02.99) IT

(71) Applicant *(for all designated States except US)*: SAICOM S.R.L. [IT/IT]; Area di Ricerca, Padriciano 99, I–34012 Trieste (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PIZZARIELLO, Andrea [IT/IT]; Via Friuli 14, I–31057 Silea (IT). STREDANSKY, Miroslav [SK/SK]; Komenskeho, 13, 900 01 Modra (SK). STREDANSKA, Silvia [SK/SK]; Komenskeho, 13, 900 01 Modra (SK). MIERTUS, Stanislav [SK/SK]; Pod Rovnicami, 27, 841 05 Bratislava (SK).

(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria 9, I–20122 Milano (IT).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PH–SENSITIVE AMPEROMETRIC BIOSENSOR

(57) Abstract

The present invention describes a new electrochemical biosensor comprising (i) a biocatalyst producing a pH change when interacting with the analyte to be determined and (ii) a compound exhibiting different redox properties both in its protonated and non–protonated forms (pH–sensitive redox compound). The elements described above are integrated in a biosensor system composed of a working electrode and a reference electrode connected to an ammeter. When the analyte is present, the system produces a current change that is proportional to the concentration of the analyte. The biosensors described herein can be used in the accurate detection of a wide range of analytes. They can be used in diagnostics, industrial processes, food and feed quality control, biotechnology, pharmaceutical industry, environmental monitoring and so on.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
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BG	Bulgaria
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BR	Brazil
BY	Belarus
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CG	Congo
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CI	Côte d'Ivoire
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DK	Denmark
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ES	Spain
FI	Finland
FR	France
GA	Gabon
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LT	Lithuania
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MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
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PT	Portugal
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SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
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TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/EP 0 0 / 0 0 4 5 5
International Application No.

2 1 JAN 2000 (21.01.2000)
International Filing Date

EUROPEAN PATENT OFFICE
PCT INTERNATIONAL APPLICATION
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 1498PTWO

Box No. I TITLE OF INVENTION

PH-SENSITIVE AMPEROMETRIC BIOSENSOR

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SAICOM S.r.l.
Area di Ricerca
Padriciano 99
34012 TRIESTE - ITALY

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: IT

State *(that is, country)* of residence: IT

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PIZZARIELLO Andrea
Via Friuli 14
31057 SILEA (Province of TREVISO) - ITALY

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: IT

State *(that is, country)* of residence: IT

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GERVASI Gemma
NOTARBARTOLO & GERVASI S.p.A.
Corso di Porta Vittoria 9
20122 MILAN - ITALY

Telephone No. +39 02541799.1

Facsimile No. +39 0254179920

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

See Notes to the request form

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

STREDANSKY Miroslav
Komenskeho 13
90001 MODRA - SLOVAK REPUBLIC

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: SK

State *(that is, country)* of residence: SK

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

STREDANSKA Silvia
Komenskeho 13
90001 MODRA - SLOVAK REPUBLIC

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: SK

State *(that is, country)* of residence: SK

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MIERTUS Stanislav
Pod Rovnicami 27
84105 BRATISLAVA - SLOVAK REPUBLIC

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: SK

State *(that is, country)* of residence: SK

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ AE United Arab Emirates
☒ AL Albania
☒ AM Armenia
☒ AT Austria
☒ AU Australia
☒ AZ Azerbaijan
☒ BA Bosnia and Herzegovina
☒ BB Barbados
☒ BG Bulgaria
☒ BR Brazil
☒ BY Belarus
☒ CA Canada
☒ CH and LI Switzerland and Liechtenstein
☒ CN China
☒ CR Costa Rica
☒ CU Cuba
☒ CZ Czech Republic
☒ DE Germany
☒ DK Denmark
☒ DM Dominica
☒ EE Estonia
☒ ES Spain
☒ FI Finland
☒ GB United Kingdom
☒ GD Grenada
☒ GE Georgia
☒ GH Ghana
☒ GM Gambia
☒ HR Croatia
☒ HU Hungary
☒ ID Indonesia
☒ IL Israel
☒ IN India
☒ IS Iceland
☒ JP Japan
☒ KE Kenya
☒ KG Kyrgyzstan
☒ KP Democratic People's Republic of Korea
☒ KR Republic of Korea
☒ KZ Kazakhstan
☒ LC Saint Lucia
☒ LK Sri Lanka
☒ LR Liberia
☒ LS Lesotho
☒ LT Lithuania
☒ LU Luxembourg
☒ LV Latvia
☒ MA Morocco
☒ MD Republic of Moldova
☒ MG Madagascar
☒ MK The former Yugoslav Republic of Macedonia
☒ MN Mongolia
☒ MW Malawi
☒ MX Mexico
☒ NO Norway
☒ NZ New Zealand
☒ PL Poland
☒ PT Portugal
☒ RO Romania
☒ RU Russian Federation
☒ SD Sudan
☒ SE Sweden
☒ SG Singapore
☒ SI Slovenia
☒ SK Slovakia
☒ SL Sierra Leone
☒ TJ Tajikistan
☒ TM Turkmenistan
☒ TR Turkey
☒ TT Trinidad and Tobago
☒ TZ United Republic of Tanzania
☒ UA Ukraine
☒ UG Uganda
☒ US United States of America
☒ UZ Uzbekistan
☒ VN Viet Nam
☒ YU Yugoslavia
☒ ZA South Africa
☒ ZW Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

☐ .....
☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

See Notes to the request form

Sheet No. 4

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (04 02 1999)* 4th February 1999	MI99A000210	ITALY		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s):

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):* Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	4
description (excluding sequence listing part)	:	19
claims	:	3
abstract	:	1
drawings	:	8
sequence listing part of description	:	
Total number of sheets	:	~~33~~ 35

This international application is **accompanied by** the item(s) marked below:

1. ☐ fee calculation sheet
2. ☒ separate signed power of attorney two forms
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: accompanyin gletter

4/** RO/EP

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

GERVASI Gemma

Milan, 17th January 2000

For receiving Office use only

1. Date of actual receipt of the purported international application: 21 JAN 2000 (21.01.00)
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

NOTARBARTOLO & GERVASI
RECEIVED
21 MAG. 2001

From the INTERNATIONAL BUREAU MILANO

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

GERVASI, Gemma
Notarbartolo & Gervasi S.p.A.
Corso di Porta Vittoria 9
I-20122 Milano
ITALIE

Date of mailing (day/month/year) 10 May 2001 (10.05.01)	
Applicant's or agent's file reference 1498PTWO	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/EP00/00455	21 January 2000 (21.01.00)	04 February 1999 (04.02.99)

Applicant
SAICOM S.R.L. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BR,BY,CH,CR,CU,DK,DM,EE,ES,FI,GB,GD,GE,GH,
   GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,MX,PT,SD,
   SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Zakaria EL KHODARY

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
GERVASI, Gemma
Notarbartolo & Gervasi S.p.A.
Corso di Porta Vittoria 9
I-20122 Milano
ITALIE

NOTARBARTOLO & GERVASI MILANO
RECEIVED
21 AGO. 2000

Date of mailing (day/month/year) 10 August 2000 (10.08.00)	
Applicant's or agent's file reference 1498PTWO	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/EP00/00455	21 January 2000 (21.01.00)	04 February 1999 (04.02.99)

Applicant
SAICOM S.R.L. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   10 August 2000 (10.08.00) under No. WO 00/46393

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year)	IMPORTANT NOTICE
10 August 2000 (10.08.00)	
Applicant's or agent's file reference	**International application No.**
1498PTWO	PCT/EP00/00455

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/00455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KULYS J. ET AL.: "Methylene-Green-mediated carbon paste glucose sensor" ELECTROANALYSIS, vol. 7, no. 1, 1995, pages 92-94, XP000916136 DE	1-3,5-7, 9-13,16
Y	the whole document ---	14,15
X	CHI Q. ET AL.: "Electrocatalytic oxidation of reduced nicotinamide coenzymes at Methylene Green-modified electrodes and fabrication of amperometric alcohol biosensors" ANAL.CHIMICA ACTA, vol. 285, 1994, pages 125-133, XP000916118 NL the whole document ---	1-3,5-7, 9-13,16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2000	18/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luzzatto, E

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	QIAN J ET AL: "An amperometric new methylene blue N-mediating sensor for hydrogen peroxide based on regenerated silk fibroin as an immobilization matrix for peroxidase;" ANAL.BIOCHEM.;(1996) 236, 2, 208-14 CODEN: ANBCA2 ISSN: 0003-2697, XP000916088 Univ.Fudan;Univ.Shanghai abstract	1
X	LOBO CASTANON M J ET AL: "Amperometric detection of ethanol with poly-(o-phenylenediamine)-modified enzyme electrodes;" BIOSENSORS BIOELECTRON.;(1997) 12, 6, 511-20 CODEN: 2026D ISSN: 0956-5663, XP000916095 Univ.Oviedo the whole document	1-3, 6-13,16
Y	EP 0 125 139 A (GENETICS INT INC) 14 November 1984 (1984-11-14) page 10, line 6 -page 14, line 21; claims 9,26	14,15
X	WANG J. ET AL.: "Amperometric biosensing of organic peroxides with peroxidase-modified electrodes" ANAL. CHIMICA ACTA, vol. 254, 1991, pages 81-88, XP000916119 NL the whole document	1-3, 6-13,16
X	WO 91 16630 A (OPTICAL SYSTEMS DEV PARTNERS) 31 October 1991 (1991-10-31) claims	1-3, 5-13,16
A	KULYS J. ET AL.: "Glucose biosensor based on the incorporation of Meldola Blue and glucose oxidase within carbon paste" ANAL. CHIMICA ACTA, vol. 288, 1994, pages 193-196, XP000916117 nl the whole document	1-3, 5-13,16
A	GORTON L. ET AL.: "Amperometric glucose sensors basd on immobilised glucose-oxidizing enzymes and chemically modified electrodes" ANAL. CHIMICA ACTA, vol. 249, 1991, pages 43-54, XP000916116 NL abstract	1-3,7

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3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	STREDANSKY M ET AL: "Amperometric pH -sensing biosensors for urea, penicillin, and oxalacetate" ANALYTICA CHIMICA ACTA, (30 JUN 2000) VOL. 415, NO. 1-2, PP. 151-157. PUBLISHER: ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS. ISSN: 0003-2670., XP000916144 the whole document	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/00455

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9116630 A	31-10-1991	NONE	

PATENT COOPERATION TREATY

23.2.01

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GERVASI, Gemma
NOTARBARTOLO & GERVASI S.P.A.
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NOTARBARTOLO & GERVASI
MILANO
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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	19.02.2001
Applicant's or agent's file reference: 1498PTWO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/00455	21/01/2000	04/02/1999

Applicant
SAICOM S.R.L. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Pedersen, C Tel.+49 89 2399 ~~8063~~ 8161

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
1498PTWO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/00455	21/01/2000	04/02/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/00		
Applicant SAICOM S.R.L. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/08/2000	19.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Luzzatto, E Telephone No. +49 89 2399 8169

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/00455

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-19	as originally filed				

Claims, No.:

7 (part),8-16	as originally filed				
1-6,7 (part)	as received on	20/12/2000	with letter of	19/12/2000	

Drawings, sheets:

1/8-8/8	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I
Basis of the opinion

1) Claim 1 has been amended by introducing a disclaimer, thus excluding oxidoreductase enzymes from its scope.
In some cases, when entering the national/regional phase, disclaimers could be considered to extend the subject-matter of the application beyond that of the application as originally filed (e.g. at the EPO, Art. 123(2) EPC, see decision of the Technical Board of Appeal T596/96).

Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 (Electroanalysis, 7, 92-94, 1995, Chi et al.)
D2 (Anal. Chimica Acta, 285, 125-33, 94, Chi et al.)
D3 (Biosens. Bioelectron., 12(6), 511-520, 97, Lobo Castañon et al.)
D4 (Wang et al., Anal. Chim. Acta, 254, 81-88, 1991)
D5 (WO-A-9116630)
D6 (Anal. Biochem., 236, 208-14, 1996, Qian et al.)
D7 (Anal. Chimica Acta, 288, 193-96, 1994, Kulys et al.)
D8 (Anal. Chimica Acta, 249, 43-54, 1991, Gorton et al.)

1) Documents D1-D4 and D6-D8 disclose the use of oxidoreductase enzymes in combination with a redox mediator. They therefore do not anticipate the subject-matter of claim 1.
D5 (WO-A-9116630) (p. 17, l. 21-p. 18, l. 16, claims) discloses a biosensor comprising a biocatalyst and a pH-sensitive compound, e.g. methylene blue. Although it mentions the possibilty that the biocatalyst could be urease, alkaline phosphatae, ß-lactamase or ß-galactosidase (see p. 12, l. 4-12,) no specific embodiments are comprising theuse of the said enzymes, or of any other non-oxidoreductade enzyme, are disclosed. Moreover, the use of a mediator is only described in combination with an oxidoreductase, and the mediator acts as a

catalyst for the electron transfer reaction. No other possible iuse of the mediators is taught.
Hence, D5 does not anticipate the subject-matter of claim 1either.
Novelty for the subject-matter of claim 1 is thus to be acknowledged (Art. 33(2) PCT). The same applies to claims 2-16 which are, directly or indirectly, dependent thereon.

2) As discussed in item 1 above, D5 is the only document which mentions enzymes which do not fall within this category. However, it falls short of suggesting their use in combination with one of the redox mediators used in combination with oxidoreductase enzymes.
No combination of D5 with any of the other the available documents would lead in an obvious way the skilled person to the subject-matter of claim 1, since they are all based on the feature that the mediator undergoes a redox reaction and in no way do they hint at the possibility of exploiting the pH-sensitivity of the said mediator. Hence their use is limited to the combination with oxidoreductase enzymes.
An inventive step for the subjct-matter of claim 1 is thus to be acknowledged (Art. 33(3) PCT).
The same applies to claims 2-16 dependent directly or inderectly on claim 1.

3) The priority of the present application appears to be valid: therefore, the paper by Stredansky et al. cited in the Search Report, does not belong to the state of the art as defined in R. 64.1 PCT.

Re Item VII
Certain defects in the international application

1) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D10 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII
Certain observations on the international application

1) Claim 2 relates to immunoproteins and nucleic acids whose use as biocatalyst is not shown to any extent in the description. In view of the fact that these molecules are seldom used as biocatalyst (and only few specific kinds thereof show such an activity (ribozymes, catalytic antibodies)) the skilled person would need some guidance as to the use of these speicifc molecules in a biosensor. Claim 2, therefore lacks support in the description (art. 6 PCT) and its subject-matter contravenes the requirements of Art. 5 PCT.

2) Claim 2 relates also to "...extracts, fractions, fragments, homogenates, lysates thereof." without however clarifying to which of the entities listed before this sentence the term "thereof" should relate. Hence claim 2 lacks clarity (Art. 6 PCT).

CLAIMS

1. An amperometric biosensor system for the detection of analytes comprising:

a) at least one biocatalyst producing a pH change by its interaction with the analyte; said biocatalyst not belonging to the group of oxidoreductase enzymes;

b) at least one compound exhibiting different redox properties in its protonated and non-protonated forms (pH-sensitive redox compounds) selected in the group consisting of cyclic hydrocarbons, containing from 4 to 30 carbon atoms and susbstituted with at least one group selected from -OH, -SH, - NH_2, =O, =S, =NH, - OR_1, -SR_1, -NHR_1, -NR_1R_2, =NR_1, wherein R_1 and R_2 are hydrocarbon chains optionally further substituted, or selected in the group consisting of heterocyclic compounds containing from 3 to 30 carbon atoms and one or more heteroatoms selected in the group consisting of N, S, O, Se, Te, B, P, As, Sb, Si, optionally substituted with a group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR, -SR_1, -NHR_1, -NR_1R_2, =NR_1, wherein R_1 and R_2 are independent hydrocarbon chains;

c) a working electrode;

d) a reference electrode;

being said electrodes connected through an ammeter.

2. The biosensor system according to claim 1, wherein said biocatalyst is selected in the group consisting of enzymes, synzymes, cells, cell components, tissues, imunoproteins, nucleic acids and extracts, fractions, fragments, homogenates, lysates thereof.

3. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of hydrolase, transferase, lyase, ligase.

4. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of phospshorylase, decarboxylase, esterase, phosphatase, deaminase.

5. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of urease, oxalacetate decarboxylase, carbonic anhydrase, penicillinase, apyrase.

6. The biosensor system according to claim 1-5, wherein said pH-sensitive redox compound is in the form of a monomer, oligomer or polymer.

7. The biosensor system according to claims 1,-6 wherein said pH-sensitive redox

PH-SENSITIVE AMPEROMETRIC BIOSENSOR

Field of the invention

The present invention relates to the field of electrochemical analysis. It refers specifically to systems for the electrochemical detection of analytes based on the activity of biocatalysts. The object of this invention is a new group of biosensors and their use in a method for the detection of analytes.

Prior art

A biosensor is a device that embodies a biological sensing element that is either connected to or inserted into a transducer. The aim is the production of electronic signals proportional to the concentration of the specific substance that has to be determined.

The advent of biosensors has provided an interesting alternative to conventional laboratory analysis. Due to their simple manipulation, compactness and versatility of use, biosensors allow for easy performances of on-site tests. Specific and sensitive devices have been used in medical diagnostics, quality assessment of food, environmental monitoring, fermentation techniques, analytical control and so on.

Electrochemical biosensors, specifically the amperometric ones, play a significant role in the use of these detection devices.

Amperometric biosensors produce a linear signal and are featured by high sensitivity. Under favourable conditions, analyte concentrations ranging from $1x10^{-8}$ to $1x10^{-9}$ M can be detected and a dynamic range from three to four order of magnitudes can be easily obtained (G.S. Wilson, in "Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford Univ. Press, 165-179, 1987).

The first generation of amperometric biosensors is based on the oxidation of the analyte by oxidases (biocatalysts) using oxygen as an electron acceptor. As a consequence, either the reduction in the oxygen concentration or the increase in the produced hydrogen peroxide concentration are measured by an electrode in the form of current that is proportional to the analyte concentration.

In the second generation systems, the enzyme performs the first redox reaction with the substrate (the analyte) but is then reoxidised by a redox mediator as

opposed to oxygen; the mediator is then oxidised by the electrode and the corresponding amperometric signal is measured. Many examples of mediators containing biosensors are quoted in a review by Gorton (Electroanalysis, 7, 23-45, 1995).

Since the redox mediators shuttle electrons that come to the redox centre of the biocatalyst from the substrate to the working electrode, a limitation inherent in these amperometric biosensors consists in the use of the biocatalysts belonging to the oxidoreductase group. As a consequence, these biosensors can detect of a limited group of analytes.

A certain number of enzymes belonging to the groups of hydrolases, transferases, oxidoreductases, lyases, ligases, and in particular decarboxylases, phosphorylases, esterases, phosphatases, deaminases, kinases, changes the concentration of H^+ ions (by either consumption or production) by their biocatalytic interaction with a substrate and this change depends on the substrate concentration. These biocatalysts, combined with a suitable potentiometric transducer (for example the typical glass pH electrode or with the solid and liquid membrane pH electrode) are used for the implementation of potentiometric biosensors. Examples of analytes that are determined by these biosensors are urea, penicillin, glucose, malate (S.S. Kuan and G.G. Guibault, In: Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford Univ. Press, 135-152, 1987; Palleschi et al., Talanta, 41, 917-923, 1994). The disadvantages of these biosensors consist in a logarithmic response and in a low sensitivity. Their useful analytical range is generally from $1x10^{-1}$ to $1x10^{-4}$ M, exceptionally to $1x10^{-5}$ M.

Another group of potentiometric biosensors uses a combination of biocatalysts that modify their pH when interfaced with ion-sensitive field effect transistors (ISFET). ISFET are prepared with a manufacturing procedure based on silicon where the silicon nitride layer deposited on the surface is mostly used as a pH-sensitive transducer. Some examples consist in biosensors for the detection of urea, ATP, penicillin, glucose and acetylcholine (G.F. Blackburn, In: Biosensors, Fundamentals and Applications, A.P.F. Turner, I. Karube and G.S. Wilson Ed., Oxford University Press, 481-530, 1987).

The drawbacks inherent in these biosensor consist in a low sensitivity (measurable response in concentration range from $1x10^{-1}$ to $1x10^{-4}$ M), high costs and a complex manufacturing procedure.

Recently, a new group of electrochemical biosensors based on the combination of a biocatalyst that modifies the pH and a conductometric transducer (A.Q. Contractor et al., Electrochim. Acta, 39, 1321-1324, 1994; J.M. Goncalves Laranjeira et al, Anal.Lett. 30, 2189-2209,1994; Nishizawa et al., Anal. Chem., 64, 2642-2644, 1992) has been described. This new kind of biosensors exploits the pH effect on the electric properties of a conductive polymer (polyaniline, polypyrrole) deposited on the electrode surface. They consist in two platinum electrodes that are placed at a distance of several μm and covered by the conductive polymer film and an enzymatic membrane. With this kind of biosensor it is possible to detect analytes such as urea, glucose, lipids, haemoglobin and penicillin. These biosensors provide a fast response and an improved sensitivity with respect to the potentiometric biosensors (the useful analytical range goes from $1x10^{-1}$ to $1x10^{-5}$ M, in the best cases $1x10^{-6}$M); however, their sensitivity is still far from the one that can be obtained with amperometric biosensors. Moreover, they require an accurate and expensive manufacturing procedure.

As a consequence, in view of the drawbacks listed previously, it is necessary to identify alternative electrochemical biosensors with higher sensitivity and an easier manufacturing procedure.

Summary of the invention

The present invention describes a new electrochemical biosensor comprising (i) a biocatalyst producing a pH change when interacting with the analyte to be determined and (ii) a compound exhibiting different redox properties both in its protonated and non-protonated forms (pH-sensitive redox compound).

The elements described above are integrated in a biosensor system composed of a working electrode and a reference electrode connected to an ammeter. When the analyte is present, the system produces a current change that is proportional to the concentration of the analyte. The biosensors described herein can be used in the accurate detection of a wide range of analytes. They can be used in diagnostics, industrial processes, food and feed quality control, biotechnology,

pharmaceutical industry, environmental monitoring and so on.

Description of figures

Figures 1-7 show the current change dependency on pH at suitable constant potentials using several pH-sensitive redox compounds and various electrodes as described in Examples 1-7.

Figure 1: platinum electrode; dissolved hematein at the concentration of 0.5 mM (curve a) and 2.5 mM (curve b);

Figure 2: dissolved hematein; carbon paste electrode (curve a) and solid composite electrode (curve b);

Figure 3: golden electrode with methylene blue monolayer;

Figure 4: solid composite electrode; dissolved hematoxylin (curve a), dissolved quercitin (curve b), dissolved harmaline (curve c);

Figure 5: solid composite electrode with electropolymerised orto-phenylendiamine;

Figure 6: platinum electrode with electropolymerised pyrogallol;

Figure 7: solid composite electrode modified with laurylgallate;

Figures 8-16 show the calibration curves of several analytes measured with the biosensors of the invention as described in the Examples 8-17.

Figure 8: biosensor for the detection of urea, dissolved hematein, platinum electrode (curve a) or solid composite electrode (curve b);

Figure 9: biosensor for the detection of urea, dissolved hematein, solid composite electrode containing urease, in the presence of either 5 mM (curve a) or 1 mM (curve b) phosphate buffer;

Figure 10: biosensor for the detection of urea, solid composite electrode modified with alkylgallate;

Figure 11: biosensor for the detection of oxaloacetate, dissolved hematein, solid composite electrode;

Figure 12: biosensor for the detection of glucose, solid composite electrode modified with poly(ortho-phenylendiamine) film;

Figure 13: biosensor for the detection of hydrogencarbonate, dissolved hematein, platinum electrode;

Figure 14: biosensor for the detection of penicillin, dissolved hematein, platinum electrode;

Figure 15: biosensor for the detection of ATP, dissolved hematein, platinum electrode;

Figure 16: biosensor for the detection of urea, golden electrode with methylene blue monolayer.

Detailed description of the invention

The object of the present invention is an amperometric biosensor system for the detection of analytes comprising:

at least one biocatalyst producing a pH change by its interaction with the analyte to be determined;

at least one compound exhibiting different redox properties both in its protonated and non-protonated forms. Said compound will be hereinafter indicated as "pH-sensitive redox compound";

a working electrode;

a reference electrode.

The electrodes at c) and d) are connected through an ammeter.

In one embodiment of this invention, the biocatalyst (a) and the pH-sensitive redox compounds (b) are contained in the working electrode; as an alternative, one or more of these components are present in the measuring solution in which the electrodes are immersed.

The biosensor of this invention can optionally be covered with a suitable semipermeable membrane.

The working principle of these biosensors is described hereinafter. The electrodes are immersed into a measuring solution and a suitable potential is applied between them. The electrode reaction is carried out up to reaching the equilibrium between the oxidised and the reduced form of the pH-sensitive redox compound (b). This electrochemical reaction is accompanied by an electron flow measured in the form of electric current by the ammeter. Up to this stage, the biocatalyst (a) is not involved. Once the sample containing the analyte is added to the solution, the biocatalyst/analyte reaction takes place and the pH is modified accordingly; the pH variation modifies the equilibrium of the protonated/non-protonated forms of the redox compound (b). Since these forms of the redox compound exhibit different redox properties, any changes in their concentration produce a current change at

the constant potential applied. The current change is monitored by the ammeter and depends on the substrate concentration.

As regards the nature of this biocatalyst (a), it can be any biological entity capable of interacting with the analyte to be determined and causing a pH variation as a result of such interaction. As a matter of fact, any biocatalyst reacting with its normal substrate either producing or consuming H^+ ions can be used as a biocatalyst for the detection of that substrate. Suitable biocatalysts are, for example, enzymes catalyzing reactions that involve either the production or the consumption of H^+ ions; typical examples are hydrolases, oxidoreductases, transferases, lyases, ligases and preferably phosphorylases, decarboxylases, esterases, proteinases, deaminases, amidases, phosphatases, and synthetases. Other examples of biocatalysts with the same features are to be found among imunoproteins, nucleic acids, sinzymes, catalytic antibodies.

Other pH changing biocatalysts can be found among biological structures or biological aggregates such as cells or cell fragments, tissues, organelles and their fragments, fractions, homogenates, extracts, lysates.

It is possible to use one single biocatalyst or a mixture of two or more of them.

The choice of the suitable biocatalysts is determined by the nature of the analyte itself, according to the principle whereby any analyte works as a substrate for a given biocatalyst: for example, esterases are indicated for the analytical detection of esters; decarboxylases are used for the detection of carboxylic acids, deaminases for amines and so on.

Examples of preferred biocatalysts for the present invention are: urease, oxalacetate decarboxylase, glucose oxidase, carbonic anhydrase, penicillinase, apyrase for the detection respectively of urea, oxalacetate, glucose, hydrogencarbonate, penicillin, ATP.

In the biosensors of the invention, the biocatalysts (a) can be incorporated in the working electrode or otherwise can be present in the measuring solution in either a dispersed or soluble form.

The incorporation of said biocatalyst in the working electrode is particularly suited for the preparation of composite biosensors: these biosensors are especially preferred.

Said biocatalysts can also be applied onto the surface of the working electrodes. In this case, they are normally immobilized by means of physical or chemical methods. The preferred methods for immobilization consist in one or more among: covering with a semipermeable membrane, entrapping in a polymer or in a gel layer, crosslinking with bifunctional agents, covalent binding, adsorption, and immobilization in the outer membrane.

The biocatalyst is normally placed in the measuring solution by dissolving the biocatalyst in the solution or by dispersing it homogeneously. This is particularly indicated for disposable thick-layer biosensors, where the biocatalyst is dissolved in the whole volume of the sample added. It is devised specifically for biosensors that determine polymeric analytes since it avoids steric hindrances that could occur when the biocatalyst is immobilized. Another possible way of placing the biocatalyst in the biosensor of the invention consists in its immobilization in a small bioreactor inserted in front of the working electrode when the flow system is applied.

When the activity of the biocatalyst requires the presence of a cofactor, for example a coenzyme or an activator, the biosensors of this invention include also said cofactor. The cofactor is preferably placed together with the biocatalyst, i.e. they are either placed onto the electrode surface, or in the electrode body or in the solution.

A further element of the biosensor system according to the present invention is represented by the pH-sensitive redox compound (b). These are compounds that are present in solution in equilibrium beween the protonated and the non-protonated form having different redox potentials.

The pH-sensitive redox compounds are selected in the group consisting of cyclic hydrocarbons containing from 4 to 30 carbon atoms and susbstituted with at least one group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR_1, -SR_1, -NHR_1, -NR_1R_2, =NR_1, where R_1 and R_2 are hydrocarbon chains optionally further substituted, or selected in the group consisting of heterocyclic compounds containing from 3 to 30 carbon atoms and one or more heteroatoms selected in the group consisting of N, S, O, Se, Te, B, P, As, Sb, Si, optionally substituted with a group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR, -SR_1, -NHR_1, -

NR_1R_2, $=NR_1$, where R_1 and R_2 are independent hydrocarbon chains. These compound can be selected in the form of either monomer, or oligomer or polymer. The above mentioned compounds can be used either alone or in a mixture with one or more of them.

The preferred classes of pH-sensitive redox compounds are indicators of pH (ie. hematoxylin, hematein), phenoxazine and phenothiazine dyes (i.e. methylene blue), natural antioxidants (i.e. quercitin, flavonoids, alkylgallates) polymerised ortho-phenylendiamine or para-phenylendiamine.

According to the invention, the pH-sensitive redox compound is present in the working electrode or dissolved in the measuring solution. The pH sensitive redox compounds that are water soluble are preferably added to the solution; those insoluble in water are preferably used to modify the working electrode.

When present in the working electrode, the pH-sensitive redox compound can be deposited onto its surface in a free form; otherwise, it can be chemically or physically bound (immobilized) onto the working electrode surface; or alternatively it can be a component of the body of a composite working electrode .

If the pH-sensitive redox compound is either a polymer or an oligomer, this can be prepared also *in situ* on the working electrode by chemical or physical polymerization, preferably by radical polymerization, electropolymerization or photopolymerization.

Among the redox compounds quoted above, phenothiazines dyes and poly(ortho-phenylendiamine) are particularly suited to be either physically or chemically bound to the electrode surface. Hematein, hematoxillin, phenothiazines dyes and quercitin are particularly indicated to be added to the measuring solution. Alkylgallates are preferably suited to be incorporated in the biosensor's body as components of a composite working electrode.

Several working electrodes can be used as element (c) of the biosensor system of this invention. Said working electrodes are selected in the group consisting of the typical working electrodes used in amperometry (like, for example, platinum, gold, mercury, glassy carbon electrodes) or by composite electrodes (such as for example the solid composite electrodes).

For the purpose of the present invention, by the term "solid composite electrodes"

are meant the electrodes described in WO 97/02359, hereby incorporated by reference.

Similarly, reference electrodes useful as element (d) of the biosensor of this invention are commonly available in amperometry. The preferred reference electrodes are standard calomel electrodes (SCE) and Ag/AgCl electrodes. The Ag/AgCl electrodes are particularly suitable because they can be designed in various forms like for example wire disc, layer or bar.

The working potential to be applied between the two electrodes is preferably about 0.0 mV or it is negative (versus Ag/AgCl reference electrode). The application of this potential significantly reduces possible electrochemical interferences deriving from easily oxidizable interfering compounds present in real samples.

Differently from the typical amperometric biosensors, where the measurements are carried out in strongly buffered solutions requiring a constant pH, in the present invention the measurements are carried out in non-buffered solutions or in solutions having a low buffering capacity. If a solution having low buffering capacity is used, the preferred concentration of the buffering compounds ranges from 0.5 to 20 mM. 0,005 – 0.80 M

The term "measuring solution" used in this invention is not strictly limited to systems where all components are dissolved; it also includes liquid systems where at least part of the components are contained in a homogeneously dispersed status such as suspensions, emulsions and so on. The biosensor implemented as described in the present invention can amperometrically determine many more analytes than was possible so far.

The biosensor system according to the present invention shows better performances in term of detection limit, linearity of the output signal, rapidity of response, selectivity and stability of those reported in literature. Besides the good specificity and sensitivity, a simple manufacturing procedure and a versatile design represent also relevant advantages of the biosensor system of the present invention. The biosensor's sensitivity described hereinafter (see Examples) ranges from 0.1 to 5 $\mu A\ mM^{-1}\ cm^{-2}$ and the detection limits range from $1x10^{-5}$ to $1x10^{-7}$ M.

The biosensors of the present invention are versatile with respect to the biocatalyst, the pH-sensitive redox compounds, the working and reference electrodes and the setting of the biosensor. They can also have a good variability in the design and can be shaped in many different forms such as for example, strips, tips and needles. Disc, tube, wire, thick layer, thin layer and other forms of the electrodes fit perfectly in the biosensor described in this invention. The preparation of microelectrodes according to the present invention is also possible. The biosensor system according to the present invention can be profitably used in human and veterinary diagnostics, industrial processes, agro-food industry, biotechnology, pharmaceutical industry, environmental monitoring and so on. All these possible uses are included in the present invention.

A further embodiment of the present invention concerns a method for the determination of the analytes concentration characterised by the use of the new biosensors described previously.

A preferred method for the determination includes the following steps:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) measuring a background current;

(d) adding to the solution the sample containing the analyte to be determined;

(e) measuring the current change that is proportional to the analyte concentration;

(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

Step (f) is added so as to eliminate possible interferences. The blank electrode differs from a normal working electrode as described so far, only in as much as it either contains said biocatalysts in a non-active form or it does not contain them at all. The procedure for obtaining a current change measured with the blank electrode is the same as the one described in steps (a)- (e).

All readings are carried out when the sample is uniformly diluted in the measuring solution and the signal is stable.

As described above, the invention is compatible with several biosensor designs, such as tips, needles, strips and so on. Some of these forms (see strip biosensor) work in absence of a measuring solution and react immediately upon contact with

the sample containing the analyte. This contact occurs for example when a drop of the sample containing the analyte is added to the biosensor, on the biosensor or by plunging the biosensor itself in the solution. In these cases, the method for the detection of the analyte is modified in the following way:

(a) applying a suitable potential between the electrodes;

(b) measuring a background current;

(c) contacting the biosensor with the sample containing the analyte;

(d) measuring a current change that is proportional to the analyte concentration;

(e) optionally subtracting the current change measured with a blank electrode from the value obtained in (d).

The methods described above can be either qualitative (they determine the presence of the analyte in the solution) or quantitative (they determine the analyte concentration) since the current change is proportional to the analyte concentration.

So far, the biocatalyst has been defined to react positively with the analyte and thereby cause a pH change. In a further embodiment of this invention, the system identifies the presence of an analyte that is an inhibitor of the biocatalyst, thereafter called inhibiting-analyte. In this case, the interaction turns out to be negative and the current change depending on the extent of the inhibition will be proportional to the inhibiting-analyte concentration.

This aspect further broadens the range of analytes that can be identified with the biosensors of the present invention; each substance acting as the inhibitor of a pH-changing biocatalyst can be identified in this way.

With the purpose of implementing this aspect of the invention, the measurement method is partly modified by adding the normal substrate of the biocatalyst to the system before introducing the sample containing the inhibiting-analyte that has to be tested. As a consequence, the method comprises the following steps:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) adding the substrate of said biocatalyst to the measuring solution;

(d) measuring a background current;

(e) adding to the solution the sample containing the inhibiting-analyte to be

determined;

(f) measuring a current change that is proportional to the inhibiting-analyte concentration;

(g) optionally subtracting the current change measured with a blank electrode from the value obtained in (f).

If the biosensor's design (e.g. strip biosensor) allows to work in absence of a measuring solution, then the above method is modified as follows:

(a) applying a suitable potential between the electrodes;

(b) adding the substrate of said biocatalyst;

(c) measuring a background current;

(d) contacting the biosensor with the sample containing the inhibiting-analyte;

(e) measuring a current change that is proportional to the inhibiting-analyte concentration;

(f) optionally subtracting from the value obtained in (d) the current change measured with a blank electrode.

Step (c) is carried out either by adding a drop of the sample containing the inhibiting-analyte to the biosensor or by immersing the sample in the solution.

This method can be further used to determine the enzymatic activities. In such case, the current changes must be measured as time-dependent.

The present invention will now be illustrated with the following experimental examples, having no limitative function.

EXPERIMENTAL PART

EXAMPLE 1

Current change variation with pH in the presence of dissolved hematein by using a platinum electrode

Hematein (Fluka, Cat. No. 51230) is dissolved in 0.05 M phosphate buffer containing 0.1 M sodium chloride. The working platinum electrode and the SCE reference electrode are immersed in the solution and the current is measured by an Amel 559 amperometric detector (Amel Instruments, Milano, Italy) at the constant potential of 0.0 mV. The pH value decreases as 2M sulphuric acid aliquots are added and the corresponding current change is monitored. Meanwhile, the pH is measured by pH-meter (PHM 85, Radiometer, Copenhagen,

Denmark). The relationship between the current change and the pH for two concentrations of hematein (0.5 mM - curve a; and 2.5 mM - curve b) is illustrated in Figure 1.

EXAMPLE 2

Current change variation with pH in the presence of dissolved hematein by using composite electrodes

The carbon paste electrode is prepared by mixing, under vigorous stirring, 7 parts (w/w) of graphite (Fluka, Cat. No. 50870) with 3 parts (w/w) of paraffin oil (Fluka, Cat. No. 76235) in a mortar. The mixture is introduced into a plastic tube (inner diameter: 2mm) equipped with a brass rod. The solid composite electrode is prepared by mixing vigorously 2 parts (w/w) of graphite with 3 parts (w/w) of melted n-eicosane (Sigma, Cat. No. E-9752) at 45°C. This mixture is introduced into a plastic tube (inner diameter: 2mm) equipped with a brass rod. Both electrodes are smoothed with a sheet of paper before use. The electrochemical measurements are carried out as described in Example 1 with 0.5 mM hematein and the current changes obtained are reported in Figure 2 (curve a – carbon paste electrode, curve b - solid composite electrode)

EXAMPLE 3

Current change variation with pH by using a golden electrode modified with methylene blue.

The newly polished golden electrode (Amel Instruments) is immersed in a 0.5 mM methylene blue solution (Aldrich, Cat. No. 86, 124-3) for 12 hours. Then, the electrode is accurately rinsed with deionized water. The electrochemical measurements are carried out as described in Example n.1, by using a working potential of -100 mV (versus SCE). The results are reported in Figure 3.

EXAMPLE 4

Current changes variation with pH by using a solid composite electrode in the presence of dissolved hematoxylin, quercitin and harmaline.

The solid composite electrodes are prepared as described in Example 2. The pH is measured in 0.5 mM solutions of hematoxylin, quercitin, harmaline by using the buffer described in Example n. 1. The working potential for hematoxylin and quercitin is 0.0 mV (versus SCE), while for harmaline is 600 mV. The results are

illustrated in Figure 4 (curve a - hematoxylin; curve b - quercitin; curve c - harmaline).

EXAMPLE 5

Current change variation with pH by using a solid composite electrode the surface of which has been modified with a poly(ortho-phenylendiamine) film.

The solid composite electrode is prepared by mixing vigorously graphite with melted n-eicosane (weight ratio1:1) at 45°C. The mixture obtained in this way is introduced into a plastic tube (inner diameter 2mm) equipped with a brass rod. A poly-(ortho-phenylendiamine) film is deposited onto the polished electrode surface by means of electrochemical polymerization of ortho-phenylendiamine monomer (Sigma, Cat. No. P-9029) in aqueous solution. This process is carried out in the following way: the scanning of the electrode potential is repeated 30 times from -0.5 mV to 0.7 mV (versus SCE) at 50 mVs^{-1} in oxygen-free 0.1 mM acetate buffer at pH 5.0 which contains 0.5 mM ortho-phenylendiamine under inert atmosphere. The modified electrode is then thoroughly rinsed with the phosphate buffer. This biosensor is then tested at different pH values of a solution and the current change is measured according to the procedure described in Example 1. The working potential is -600 mV. The results are illustrated in Figure 5.

EXAMPLE 6

Current change variation for a platinum electrode the surface of which is modified with polypyrogallol.

The polypyrogallol film is deposited upon the newly polished surface of the platinum electrode by electrochemical polymerization of 25 mM of pyrogallol (Aldrich, Cat. Mo. 25.400-2) in aqueous solution containing 0.15 M phosphate buffer (pH 7.0) and tetraethylamonium tetrafluoroborate 0.1 M (Aldrich, Cat. No. 24, 214-4). The scanning of the potential electrode is repeated three times from 0.0 V and 1.1 V (versus SCE) at 50 mVs^{-1}. The modified electrode is then rinsed thoroughly with the phosphate buffer. This biosensor is tested at the different pH values of a solution and the current change is measured with the same procedure as described in Example n. 1. The working potential is 200 mV. The results are shown in Figure 6.

EXAMPLE 7

Variation of the current changes for a solid composite electrode modified with lauryl gallate.

The graphite powder is modified as follows: 100 mg of lauryl gallate (Fluka, Cat. No. 48660) are dissolved in 2 ml of acetone and 400 mg of modified graphite are added to the solution. The mixture is stirred up to being made homogeneous and acetone is then evaporated under forced air flow at room temperature. 100 mg of lauric acid (Fluka, Cat. Mo. 61610) and 150 mg of 2-hexadecanone (Fluka, Cat. No. 69250) are dissolved in a porcelain dish at 50 °C and stirred vigorously with 250 mg of the modified graphite. A plastic tube (inner diameter 2 mm) equipped with a brass rod is filled with this mixture; the electrode material then solidifies at room temperature. The electrode surface is smoothed with sand paper and cleansed with a sheet of common paper. The current change dependence on the pH of the electrode modified by lauryl gallate is measured with the same procedure as the one described in Example n.1. The working potential is 200 mV. The results are illustrated in Figure 7.

EXAMPLE 8

Preparation of the biosensor for the determination of urea based on a platinum electrode modified with urease and dissolved hematein

A solution (2 µl, 10 mg/ml) of urease (EC 3.5.1.5., Sigma, Cat. No. U-0376) is applied onto the surface of the platinum electrode. After drying at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000), fixed by means of an O-ring. The biosensor is immersed in 1 mM phosphate buffer (pH=7.35) containing 0.5 mM hematein and 0.1 mM sodium chloride. Hence, the biosensor is polarized at 0.0 mV (versus SCE) and a few aliquots of urea solution (5 mg/ml) are added to the measuring buffer. The relationship between the urea concentration and the current change is reported in Figure 8 (curve a).

EXAMPLE 9

Preparation of the biosensor for the determination of urea based on the solid composite electrode modified with urease and dissolved hematein.

The solid composite electrode is prepared as described in Example 2. Urease (2 µl, 10 mg) is applied onto the clean electrode surface. After drying, the electrode is

covered with a dialysis membrane (Spectra/por MWCO 6,000 - 8,000) by means of an O-ring. The biosensor is then immersed in 1mM phosphate buffer (pH = 7.35) containing 0.5 mM hematein and 0.1 M sodium chloride. The electrode is then polarized at 0.0 mV (versus SCE). A few aliquots of urea standard solutions (5mg/ml) are added to the measuring buffer. The relationship between the urea concentration and the current change is shown in Figure 8 (curve b).

EXAMPLE 10

Preparation of the biosensor for detection of urea based on the bulk modified solid composite electrode and dissolved hematein

The graphite powder is modifed in the following way: 97 mg of graphite powder are added to 0.5 ml urease aqueous solution (6 mg/ml). The mixture is accurately mixed to obtaining a homogeneous mixture and water is then gently evaporated. 50 mg of the modified graphite are mixed with 50 mg of 2-hexadecanone at 50 °C and the mixture obtained is poured into a plastic tube (inner diameter 2 mm) equipped with a brass rod; the mixture is then cooled down at room temperature. The electrode is smoothed with a sheet of paper and covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000). The biosensor is immersed in the phosphate buffer (1 or 5mM, pH 7.50) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of urea standard solution (5 mg/ml) are added to the measuring buffer. The current changes are recorded and the results are illustrated in Figure 9, where curve b) refers to 1 mM phosphate buffer.

EXAMPLE 11

Preparation of the biosensor for the determination of urea by using a solid composite electrode modified with urease and containing lauryl gallate.

The graphite powder is modified in the following way: 20 mg of lauryl gallate are dissolved in 0.5 ml of acetone and 90 mg of graphite are added to the solution. The mixture then is stirred up to making it homogeneous and acetone is evaporated under forced air at room temperature. 40 mg of 2-hexadecanone and 5 mg of stearic acid (Aldrich, Cat. No. 26, 838 - 0) are dissolved in a porcelain dish at 55 °C and mixed vigorously with 55 mg of the modified graphite quoted above. The mixture is then poured into a plastic tube (inner diameter: 2mm) equipped with

a brass rod. Urease (1µl, 30 mg/ml) is applied onto the newly cleansed electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed with an O-ring. The biosensor is immersed in 1mM phosphate buffer (pH 7.35 containing 0.1 M sodium chloride). It is then polarized at 200 mV (versus SCE). Then several aliquots of standard solutions of urea (5 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the urea concentration and the current change is illustrated in Figure 10.

This biosensor allows to perfom 30 reproducibile measurements.

This biosensor is tested after storage in dried state at temperature of 22±2°C under controlled umidity (<0.5%). After 6 month the sensitivity variation is not significant (<3%).

EXAMPLE 12

Preparation of the biosensor for the determination of oxalacetate by using a solid composite electrode modified with oxalacetate decarboxylase and dissolved hematein.

The solid composite electrode is described in Example n. 2. The oxalacetate decarboxylase (EC 4.1.1.3., ICN, Cat. No. 156007, 5,3 U) is applied onto the electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by means of an 0-ring. The biosensor is then plunged into 1 mM phosphate buffer (pH 8.0) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). Several aliquots of standard solutions of sodium oxalacetate (20 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the oxalacetate concentration and the current change is shown in Figure 11.

EXAMPLE 13

Preparation of the biosensor based on a solid composite electrode modified with glucose oxidase and covered with a poly(para-phenylendiamine) film.

The solid composite electrode with the thick poly(para-phenylendiamine) film is prepared as described in Example N.5. The glucose oxidase (EC 1.1.3.4, Sigma, Cat. No. G-7016, 2 µl, 10 mg/ml) is applied onto the electrode surface that is then rinsed and covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000)

fixed with an O-ring. The biosensor is then immersed in a phosphate buffer (1mM, pH 7.0) containing 0.1 M sodium chloride. It si then polarized at -600 mV (versus SCE). Several aliquots of glucose standard solutions (20 mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the glucose concentration and the current change is shown in Fig. 12.

EXAMPLE 14

Preparation of the biosensor for the determination of hydrogen carbonate based on a platinum electrode modified with carbonic anhydrase and dissolved hematein.

A solution of carbonic anhydrase (EC 4.2.1.1, Sigma, Cat. No. C 4831, 2400 W-A units, 2µl, 100 mg/ml) is applied onto the surface of the platinum electrode. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by means of an O-ring. The biosensor is then immersed in Tris-HCl 4 mM (pH 8.30) containing 0.5 mM hematein and sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of sodium hydrogen-carbonate standard solutions (10 mg/ml) are added to the mesauring buffer. The relationship between the concentration of hydrogen carbonate and the current change is shown in Figure 13.

EXAMPLE 15

Preparation of the biosensor based on a platinum working electrode modified with penicillinase and dissolved hematein.

A solution (2 µl, 100 mg/ml) of penicillinase (EC 3.5.2.6, Sigma, Cat. No P-0389) is applied onto the platinum electrode surface. After having dried it at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is then immersed in 1 mM phosphate buffer (pH= 7.5) containing 0.5 mM hematein and 0.1 M sodium chloride. It is then polarized at 0.0 mV (versus SCE). A few aliquots of standard solutions of benzylpenicilline sodium salt (20 mg/ml) are added to the measuring solution. The current changes are recorded. The relationship between the benzylpenicilline and the current change is illustrated in Figure 14.

EXAMPLE 16

Preparation of the biosensor for ATP determination based on a platinum working electrode modified with apyrase and dissolved hematein.

One solution (2µl, 200 mg/ml) of apyrase (EC 3.6.1.5, Sigma, Cat. No A- 6132) is applied onto the platinum electrode surface. After having dried it at room temperature, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is immersed in Tris-HCl 2 mM (pH=7.0) containing 0.5 mM of hematein and 0.25 of sodium chloride. It is then polarized at 0.0 mV (versus SCE). Several aliquots of standard solutions of ATP sodium salt (20/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the ATP concentration and the current change is illustrated in Figure 15.

EXAMPLE 17

Preparation of the biosensor for urea determination based on a golden electrode modified with methylene blue

The electrode is prepared as described in Example n. 3. Urease (3 µl, 10 mg/ml) is applied onto the electrode surface. After drying, the electrode is covered with a dialysis membrane (Spectra/Por MWCO 6,000 - 8,000) fixed by an O-ring. The biosensor is immersed in 1 mM phosphate buffer (pH = 7.50) containing 0.1 M sodium chloride. It is then polarized at -100 mV (versus SCE). A few aliquots of urea standard solution (5mg/ml) are added to the measuring buffer. The current changes are recorded. The relationship between the urea concentration and the current change is shown in Figure 16.

CLAIMS

1. An amperometric biosensor system for the detection of analytes comprising:
a) at least one biocatalyst producing a pH change by its interaction with the analyte;
b) at least one compound exhibiting different redox properties in its protonated and non-protonated forms (pH-sensitive redox compounds) selected in the group consisting of cyclic hydrocarbons, containing from 4 to 30 carbon atoms and susbstituted with at least one group selected from -OH, -SH, - NH_2, =O, =S, =NH, - OR_1, - SR_1, - NHR_1, - NR_1R_2, = NR_1, wherein R_1 and R_2 are hydrocarbon chains optionally further substituted, or selected in the group consisting of heterocyclic compounds containing from 3 to 30 carbon atoms and one or more heteroatoms selected in the group consisting of N, S, O, Se, Te, B, P, As, Sb, Si, optionally substituted with a group selected from -OH, -SH, - NH_2, =O, =S, =NH, -OR, - SR_1, - NHR_1, - NR_1R_2, = NR_1, wherein R_1 and R_2 are independent hydrocarbon chains;
c) a working electrode;
d) a reference electrode;
being said electrodes connected through an ammeter.

2. The biosensor system according to claim 1, wherein said biocatalyst is selected in the group consisting of enzymes, synzymes, cells, cell components, tissues, imunoproteins, nucleic acids and extracts, fractions, fragments, homogenates, lysates thereof.

3. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of hydrolase, oxydoreductase, transferase, lyase, ligase.

4. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of phospshorylase, decarboxylase, esterase, phosphatase, deaminase.

5. The biosensor system according to claim 2, wherein said enzyme is selected in the group consisting of urease, oxalacetate decarboxylase, glucose oxidase, carbonic anhydrase, penicillinase, apyrase.

6. The biosensor system according to claim 1-5, wherein said pH-sensitive redox compound is in the form of a monomer, oligomer or polymer.

7. The biosensor system according to claims 1-6 wherein said pH-sensitive redox

compound (b) is selected among the pH indicators, phenoxazines and phenothiazines dyes, and natural antioxidants.

8. The biosensor system according to claim 7, wherein said pH-sensitive redox compound (b) is selected in the group consisting of hematoxylin, hematein, methylene blue, quercitin, flavonoids, alkyl gallates, polymerized ortho-phenylenediamine or para-phenylendiamine.

9. The biosensor system according to claims 1-8, wherein said working electrode (c) is a solid composite electrode, or platinum electrode , or gold electrode, or mercury electrode or glassy carbon electrode.

10. The biosensor system according to claims 1-9 wherein said reference electrode (d) is selected in the group consisting of Ag/AgCl and calomel electrodes.

11. A method for the determination of analytes characterized by the use of a biosensor as claimed in claims 1-10.

12. A method according to claim 11, wherein said method consists in:

(a) placing the electrodes in a measuring solution;

(b) applying a suitable potential between the electrodes;

(c) measuring a background current;

(d) adding to the solution the sample containing the analyte to be determined;

(e) measuring the current change that is proportional to the analyte concentration;

(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

13. A method according to claim 11, wherein said method consists in:

(a) applying a suitable potential between the electrodes;

(b) measuring a background current;

(c) contacting the biosensor with the sample containing the analyte;

(d) measuring a current change that is proportional to the analyte concentration;

(e) optionally subtracting the current change measured with a blank electrode from the value obtained in (d)

14. A method according to claim 11, wherein said biocatalyst contained in the biosensor system is selected among the biocatalysts that are inhibited by said analyte, said method consisting in:

(a) placing the electrodes in a measuring solution;
(b) applying a suitable potential between the electrodes;
(c) adding the substrate of said biocatalyst to the measuring solution;
(d) measuring a background current;
(e) adding to the solution the sample containing the inhibiting-analyte to be determined;
(f) measuring a current change that is proportional the inhibiting-analyte concentration;
(g) optionally subtracting the current change measured with a blank electrode from the value obtained in (f).

15. A method according to claim 11, wherein said biocatalyst contained in the biosensor system is selected among the biocatalysts that are inhibited by said analyte said method consisting in:
(a) applying a suitable potential between the electrodes;
(b) adding the substrate of said biocatalyst;
(c) measuring a background current;
(d) contacting the biosensor with the sample containing the inhibiting-analyte system;
(e) measuring a current change that is proportional to the inhibiting-analyte concentration;
(f) optionally subtracting the current change measured with a blank electrode from the value obtained in (e).

16. Use of the biosensor system as claimed in claims 1-10 for the amperometric detection of analytes in human and veterinary diagnostics, industrial processes, agro-food industry, pharmaceutical industry, environmental monitoring.

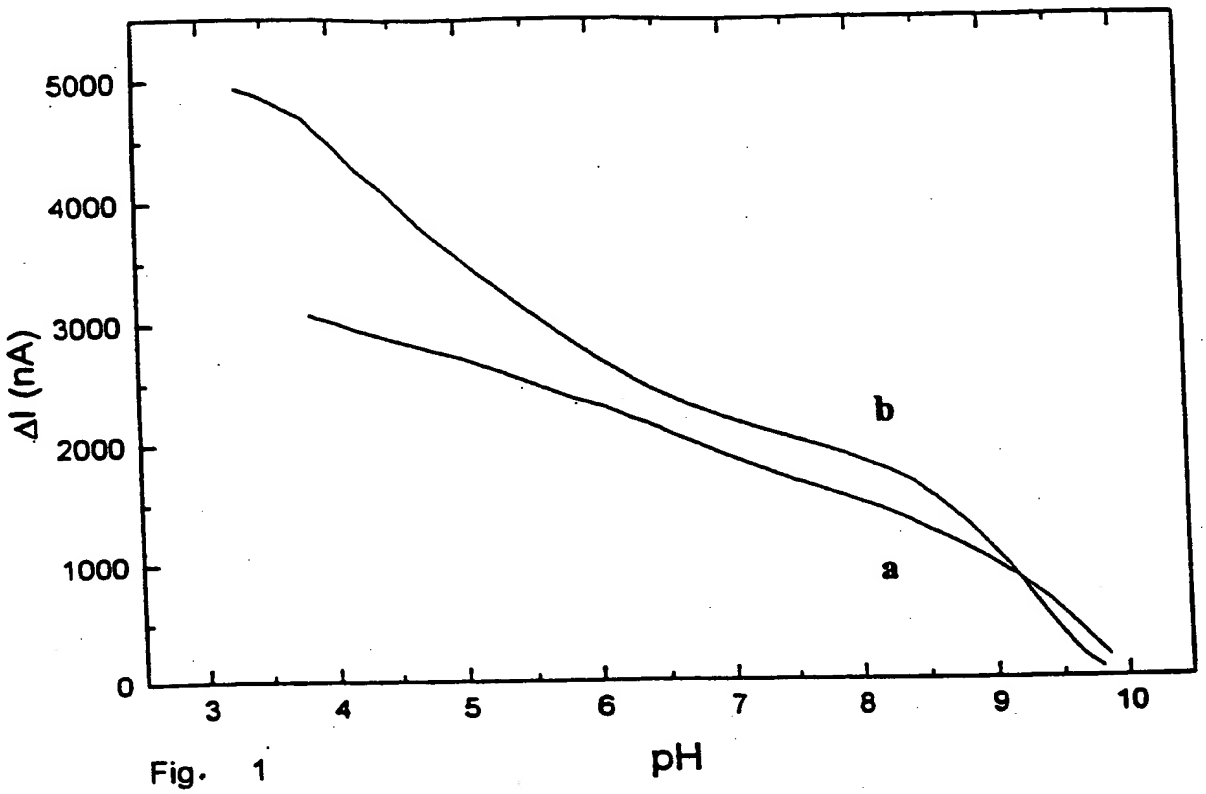
5000
4000
3000
2000
1000
0
ΔI (nA)
3
4
5
6
7
8
9
10
pH
a
b

Fig. 1

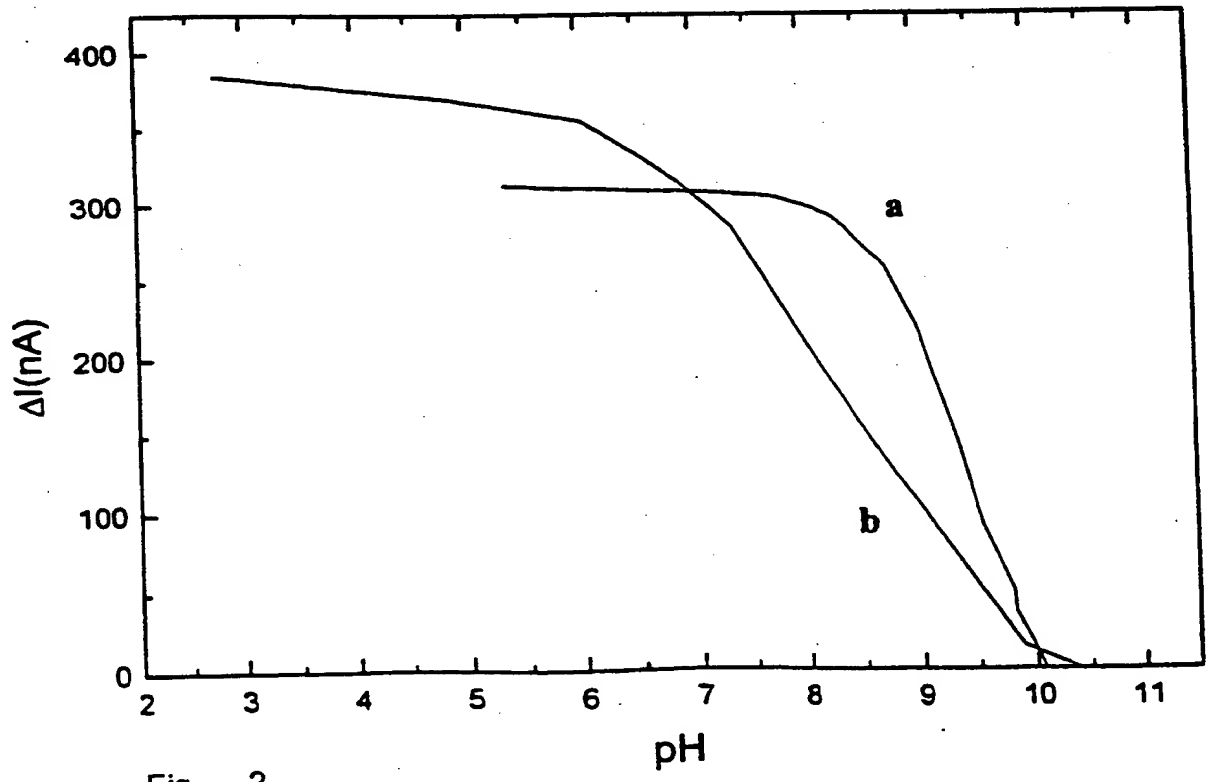
400
300
200
100
0
ΔI(nA)
2
3
4
5
6
7
8
9
10
11
pH
a
b

Fig. 2

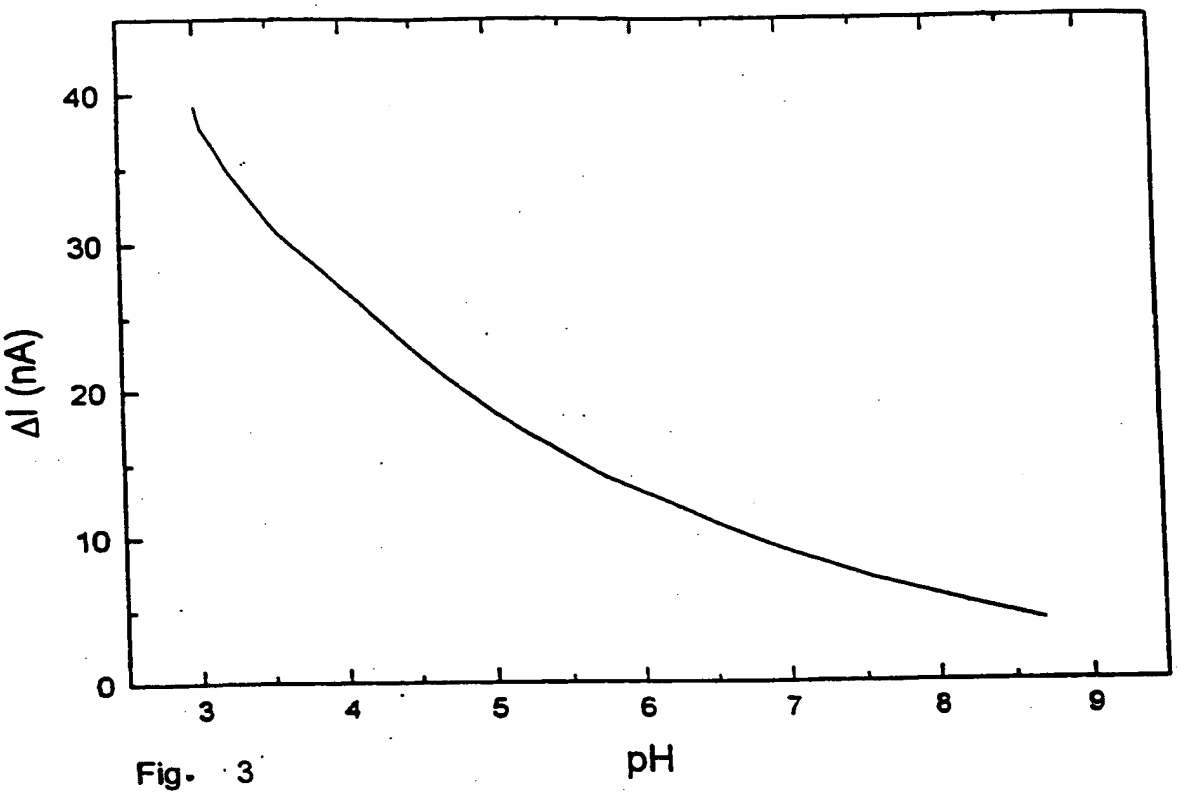
40
30
20
10
0
ΔI (nA)
3
4
5
6
7
8
9
pH

Fig. 3

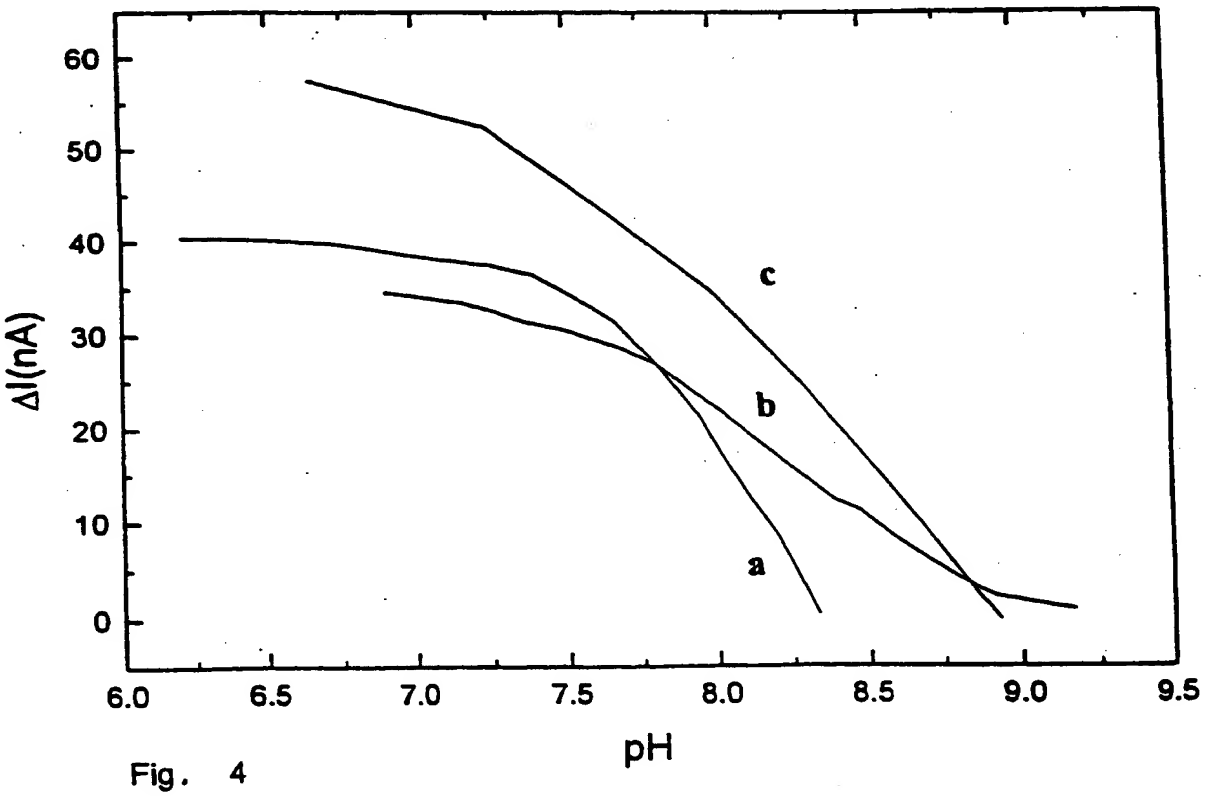
60
50
40
30
20
10
0
ΔI(nA)
c
b
a
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH

Fig. 4

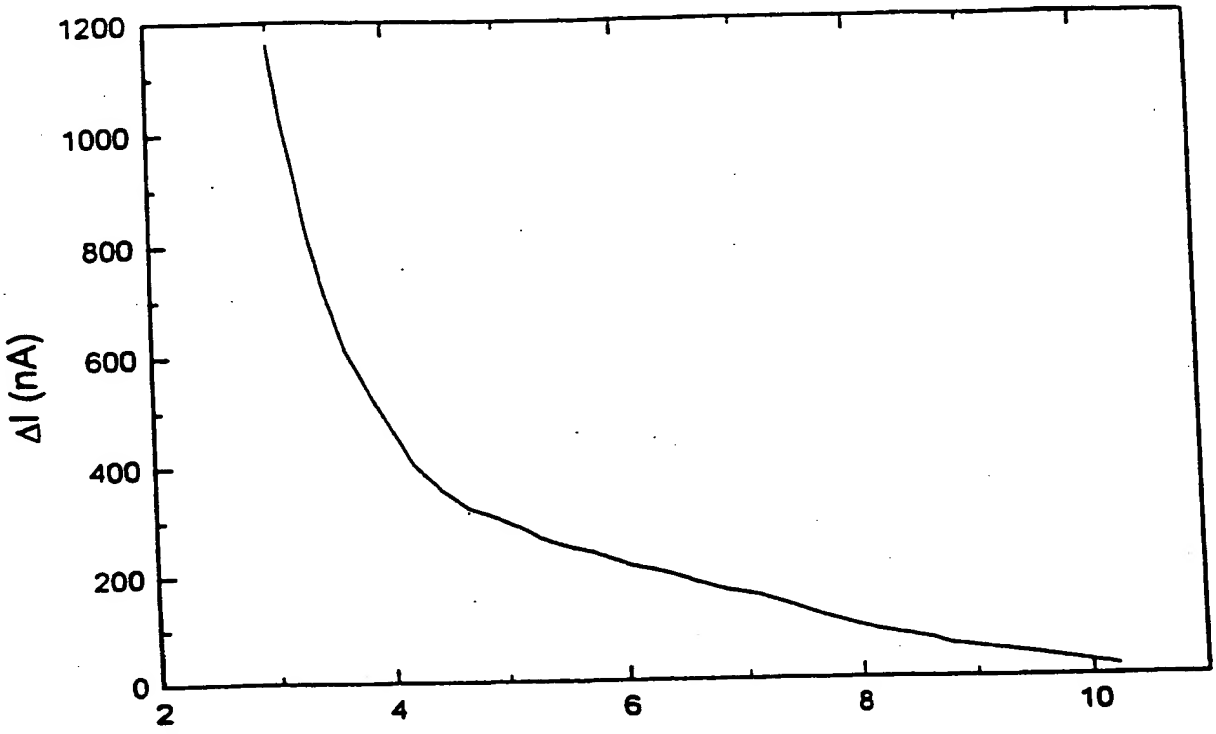
1200
1000
800
600
400
200
0
ΔI (nA)
2
4
6
8
10
pH

Fig. 5

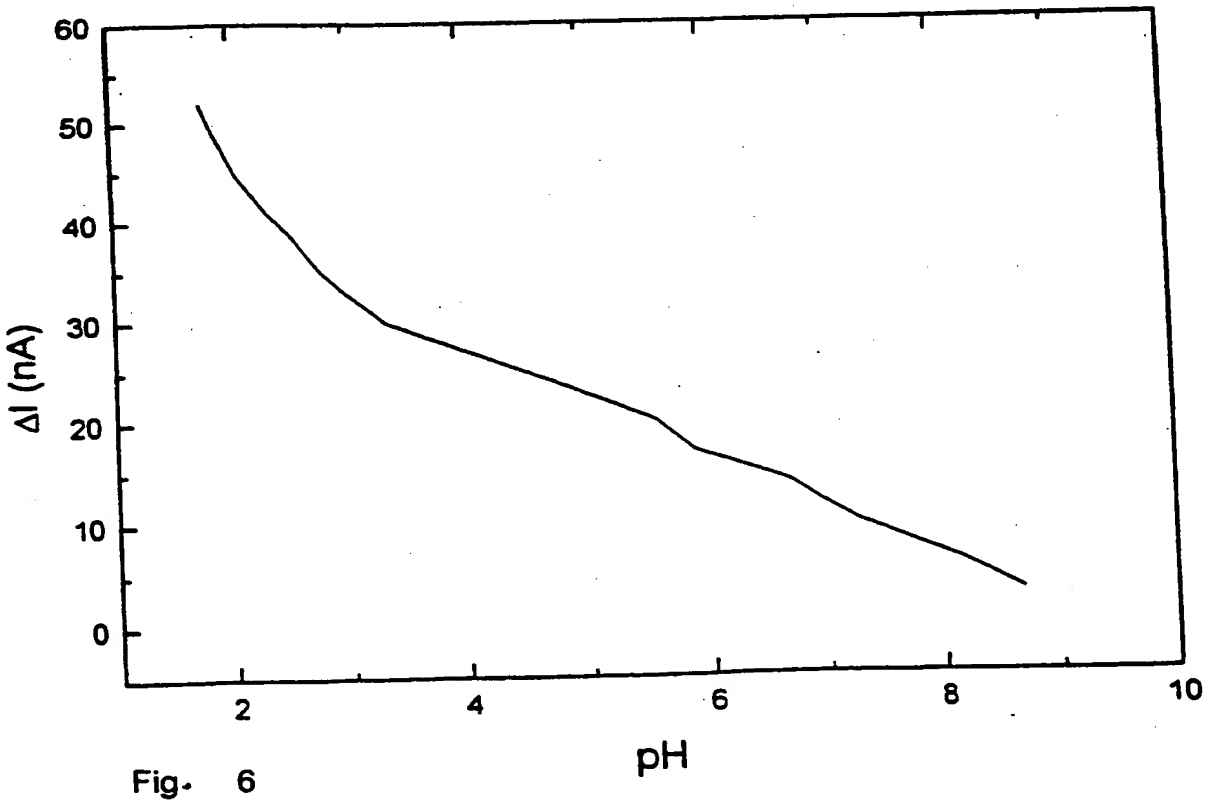
60
50
40
30
20
10
0
ΔI (nA)
2
4
6
8
10
pH

Fig. 6

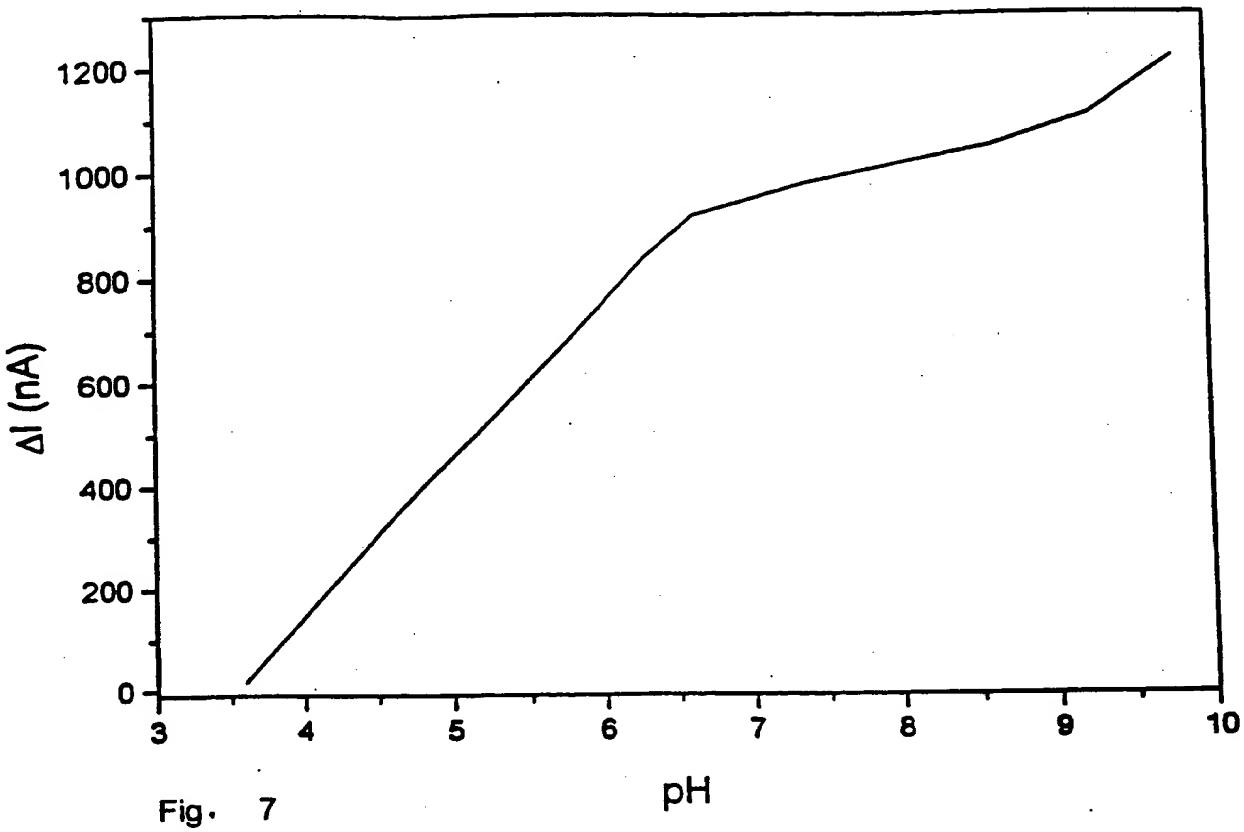
1200
1000
800
600
400
200
0
ΔI (nA)
3
4
5
6
7
8
9
10
pH

Fig. 7

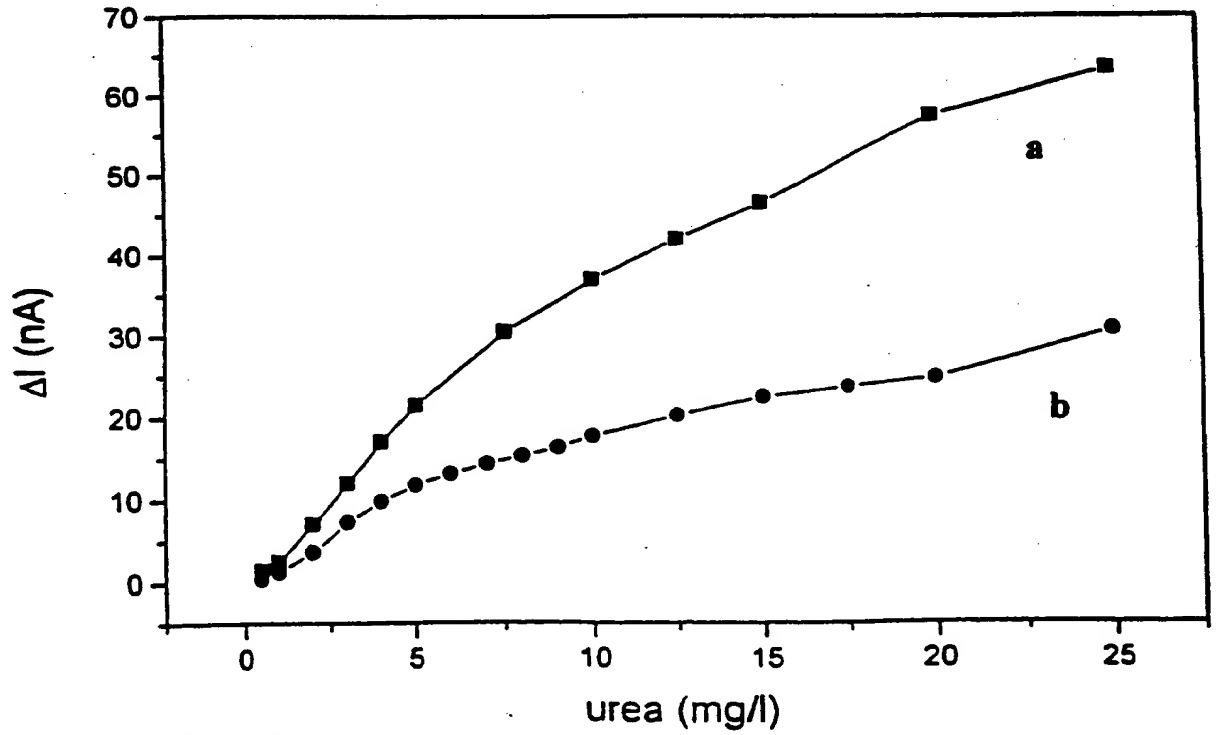
70
60
50
40
30
20
10
0
ΔI (nA)
a
b
0
5
10
15
20
25
urea (mg/l)

Fig. 8

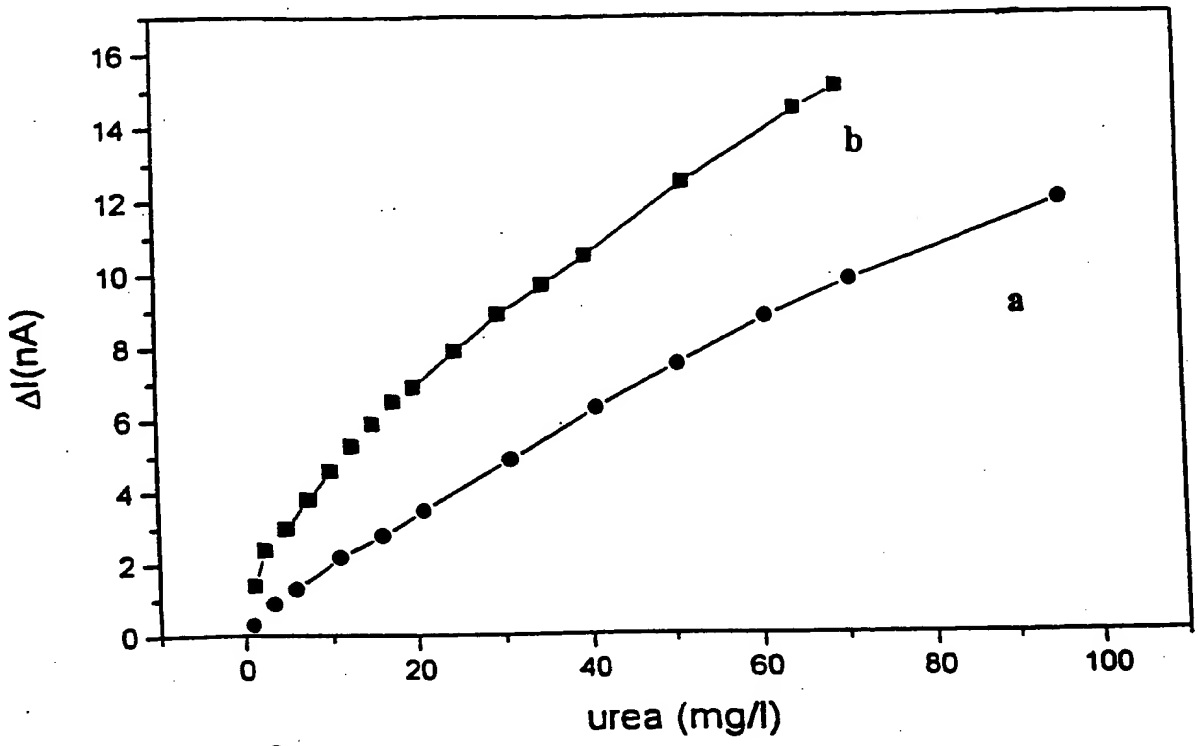
16
14
12
10
8
6
4
2
0
ΔI(nA)
0
20
40
60
80
100
urea (mg/l)
b
a

Fig. 9

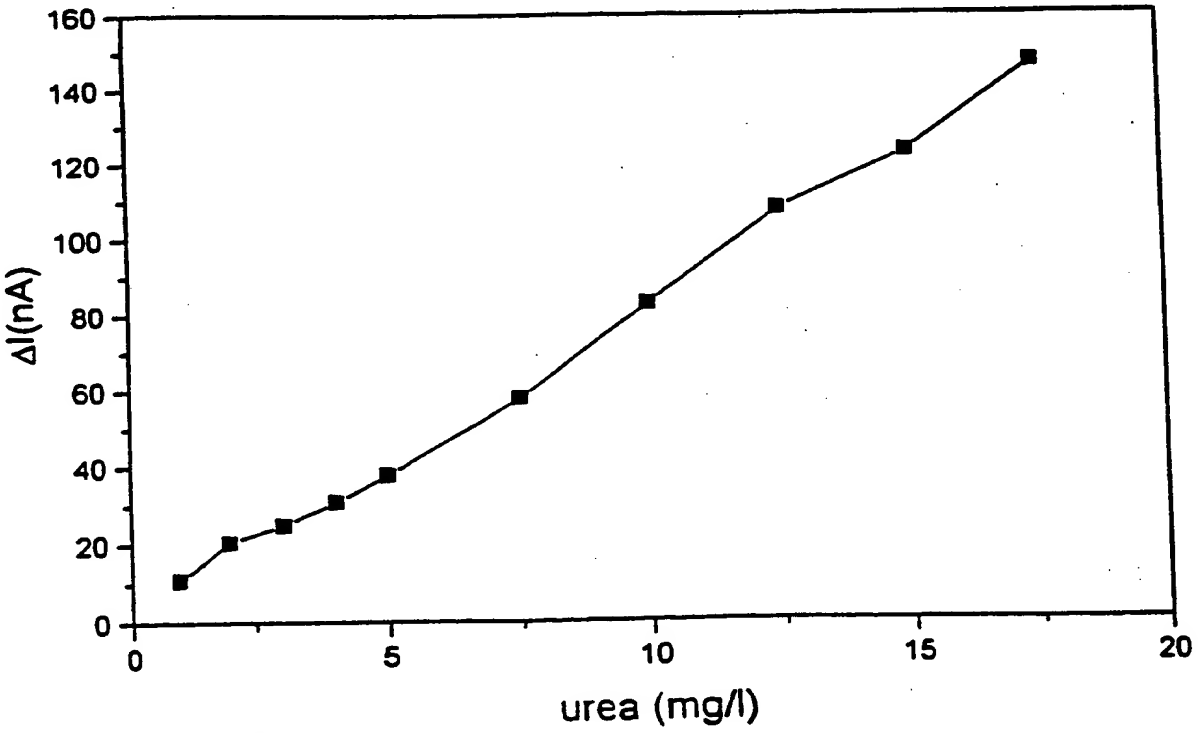
160
140
120
100
80
60
40
20
0
ΔI(nA)
0
5
10
15
20
urea (mg/l)

Fig. 10

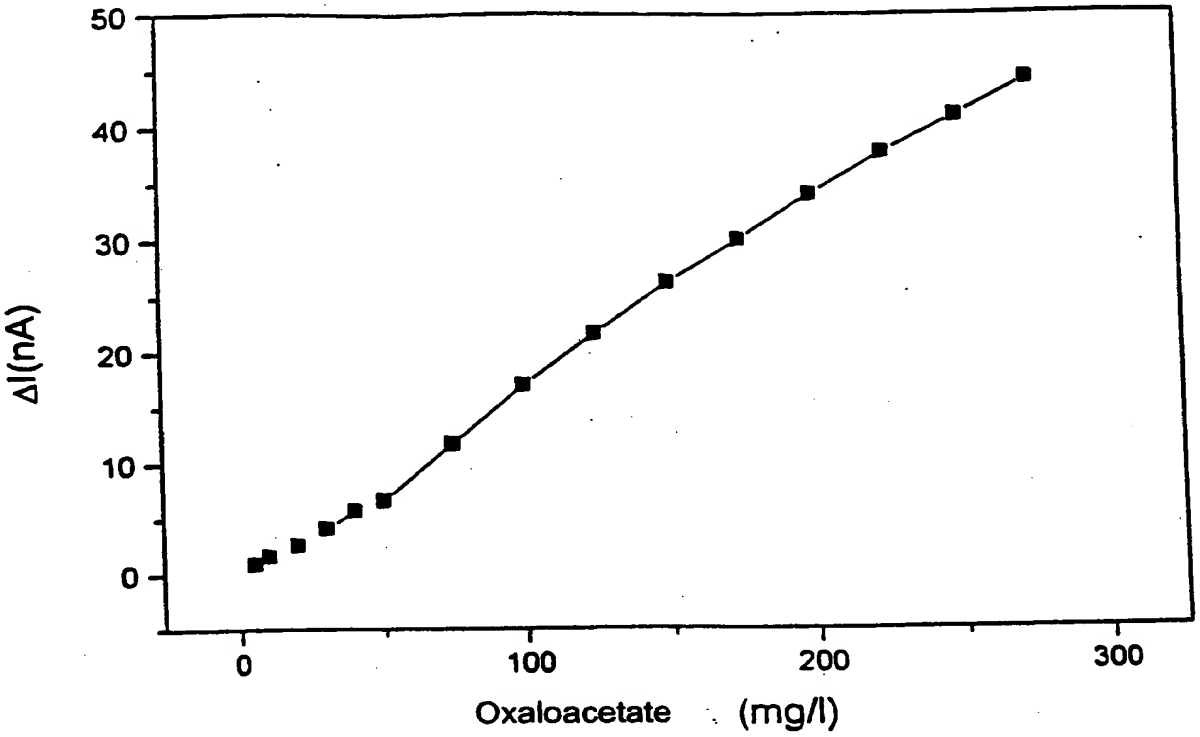
50
40
30
20
10
0
ΔI(nA)
0
100
200
300
Oxaloacetate (mg/l)

Fig. 11

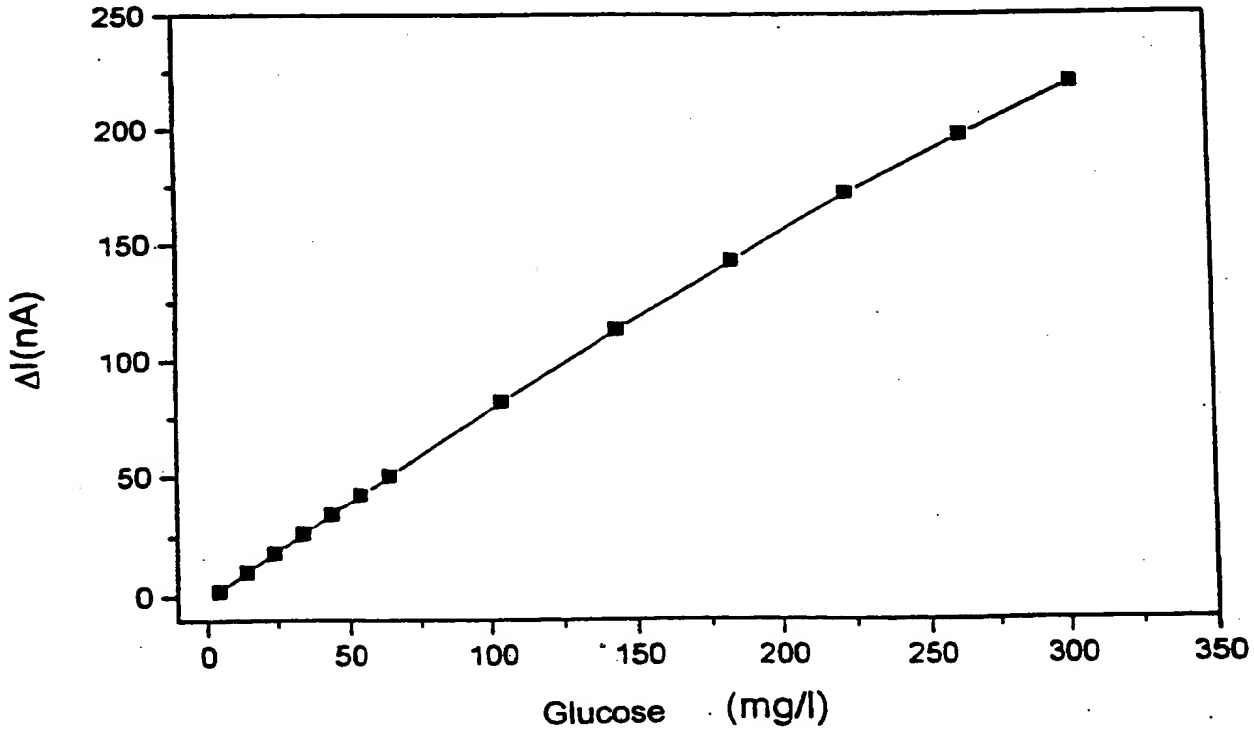
250
200
150
100
50
0
ΔI(nA)
0
50
100
150
200
250
300
350
Glucose (mg/l)

Fig. 12

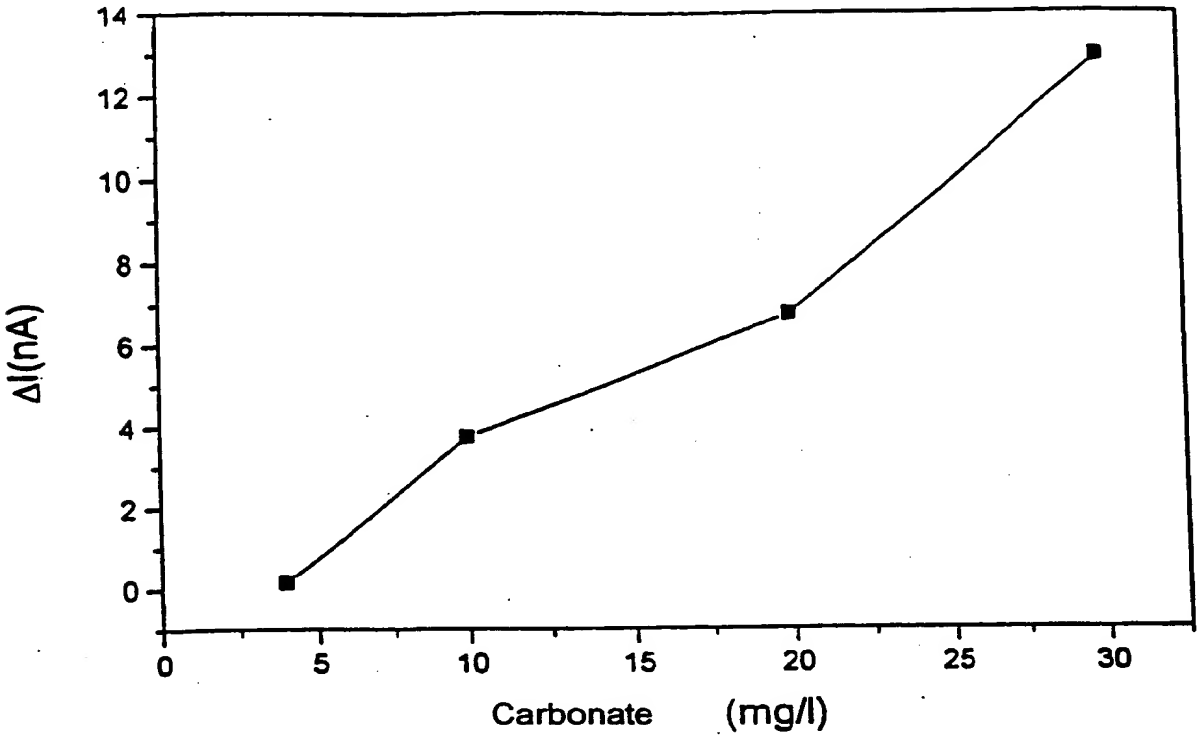
14
12
10
8
6
4
2
0
ΔI(nA)
0
5
10
15
20
25
30
Carbonate (mg/l)

Fig. 13

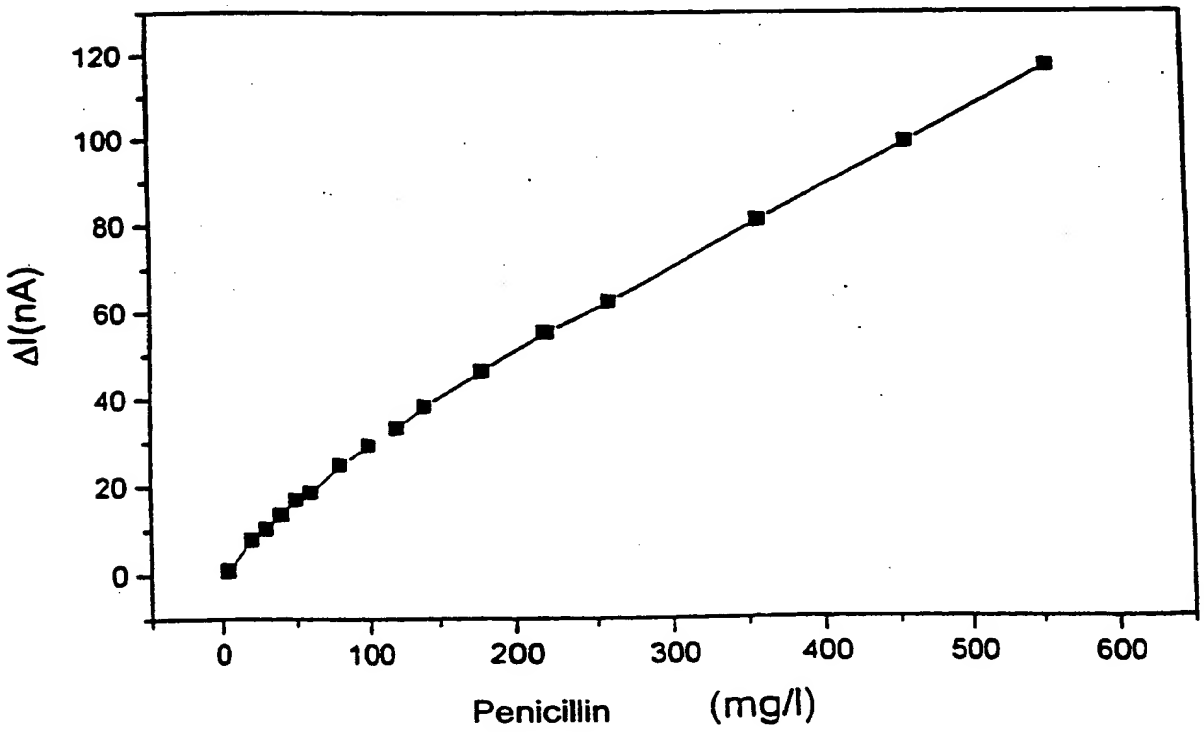
120
100
80
60
40
20
0
ΔI(nA)
0
100
200
300
400
500
600
Penicillin (mg/l)

Fig. 14

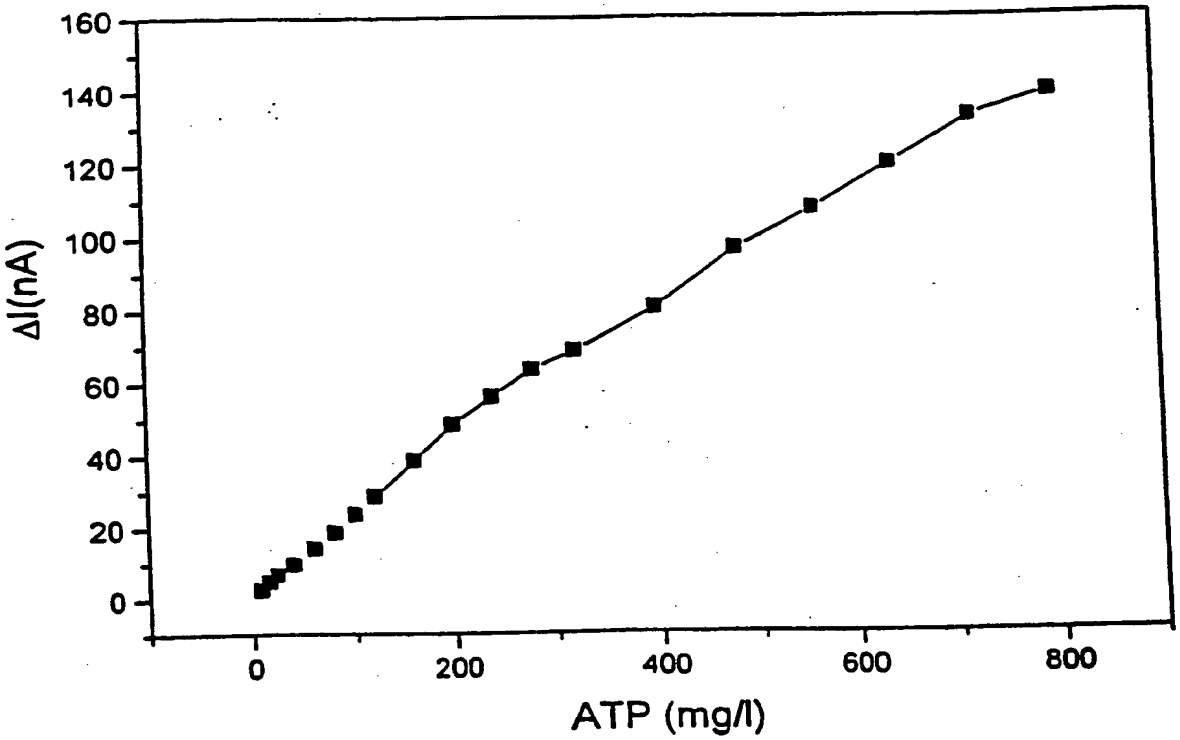
160
140
120
100
80
60
40
20
0
ΔI(nA)
0
200
400
600
800
ATP (mg/l)

Fig. 15

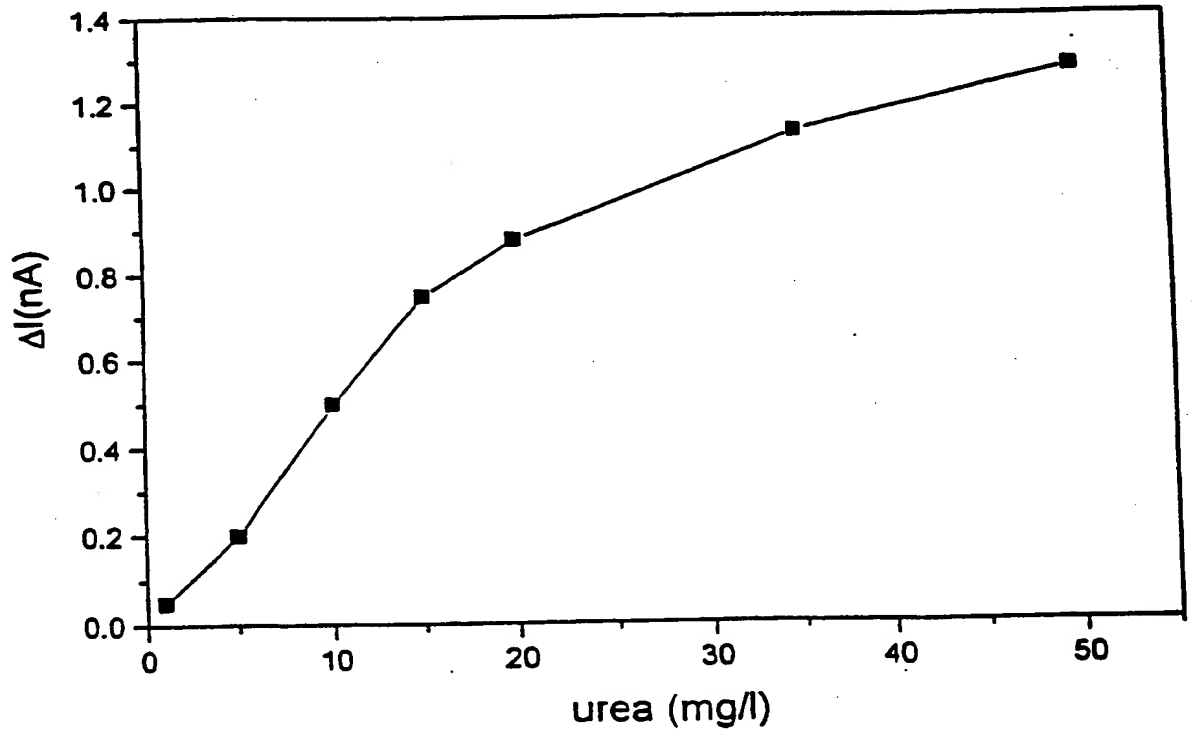
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ΔI(nA)
0
10
20
30
40
50
urea (mg/l)

Fig. 16

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/00455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KULYS J. ET AL.: "Methylene-Green-mediated carbon paste glucose sensor" ELECTROANALYSIS, vol. 7, no. 1, 1995, pages 92-94, XP000916136 DE	1-3,5-7, 9-13,16
Y	the whole document	14,15
X	CHI Q. ET AL.: "Electrocatalytic oxidation of reduced nicotinamide coenzymes at Methylene Green-modified electrodes and fabrication of amperometric alcohol biosensors" ANAL.CHIMICA ACTA, vol. 285, 1994, pages 125-133, XP000916118 NL the whole document	1-3,5-7, 9-13,16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2000	18/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luzzatto, E

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INTERNATIONAL SEARCH REPORT

I. ıational Application No
PCT/EP 00/00455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	QIAN J ET AL: "An amperometric new methylene blue N-mediating sensor for hydrogen peroxide based on regenerated silk fibroin as an immobilization matrix for peroxidase;" ANAL.BIOCHEM.;(1996) 236, 2, 208-14 CODEN: ANBCA2 ISSN: 0003-2697, XP000916088 Univ.Fudan;Univ.Shanghai abstract	1
X	LOBO CASTANON M J ET AL: "Amperometric detection of ethanol with poly-(o-phenylenediamine)-modified enzyme electrodes;" BIOSENSORS BIOELECTRON.;(1997) 12, 6, 511-20 CODEN: 2026D ISSN: 0956-5663, XP000916095 Univ.Oviedo the whole document	1-3, 6-13,16
Y	EP 0 125 139 A (GENETICS INT INC) 14 November 1984 (1984-11-14) page 10, line 6 -page 14, line 21; claims 9,26	14,15
X	WANG J. ET AL.: "Amperometric biosensing of organic peroxides with peroxidase-modified electrodes" ANAL. CHIMICA ACTA, vol. 254, 1991, pages 81-88, XP000916119 NL the whole document	1-3, 6-13,16
X	WO 91 16630 A (OPTICAL SYSTEMS DEV PARTNERS) 31 October 1991 (1991-10-31) claims	1-3, 5-13,16
A	KULYS J. ET AL.: "Glucose biosensor based on the incorporation of Meldola Blue and glucose oxidase within carbon paste" ANAL. CHIMICA ACTA, vol. 288, 1994, pages 193-196, XP000916117 nl the whole document	1-3, 5-13,16
A	GORTON L. ET AL.: "Amperometric glucose sensors basd on immobilised glucose-oxidizing enzymes and chemically modified electrodes" ANAL. CHIMICA ACTA, vol. 249, 1991, pages 43-54, XP000916116 NL abstract	1-3,7

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3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/00455

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE 3486221 T	27-01-1994
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WO 9116630 A	31-10-1991	NONE	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	STREDANSKY M ET AL: "Amperometric pH -sensing biosensors for urea, penicillin, and oxalacetate" ANALYTICA CHIMICA ACTA, (30 JUN 2000) VOL. 415, NO. 1-2, PP. 151-157. PUBLISHER: ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS. ISSN: 0003-2670., XP000916144 the whole document	1-16